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## (54) METHOD AND COMPOSITIONS FOR THE TREATMENT AND PREVENTION OF SEPTIC SHOCK.

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**EP 0 428 573 B1**

## Description

This invention relates to the use of transforming growth factor- $\beta$  in the manufacture of a medicament for the treatment of patients suffering from or at risk of septic shock caused by bacteremic infections. More particularly, this invention relates to the use of transforming growth factor- $\beta$  in the manufacture of a medicament for the prophylaxis of incipient septic shock and to the amelioration of the symptoms characteristic of acute septic shock.

Septic shock is a widespread and hazardous syndrome that frequently accompanies severe Gram-negative and, to a lesser degree, Gram-positive bacteremia. According to recent studies, in a hypothetical group of 100 patients who are bacteremic with Gram-negative organisms, the incidence of metabolic complications and shock is about 25%, while about 10% of patients with Gram-positive bacteremia (especially from *S. aureus* infections) develop shock.

The presence of bacteremic shock dramatically increases morbidity and mortality. In cases in which the inciting infection is localized, shock is associated with a 50% mortality. Frank shock accompanying systemic bacteremia is characterized by greater than 70% mortality. In addition, recovery from the effects of shock and bacteremia typically requires long-term intensive care at great expense.

Gram-negative rods such as Enterobacteriaceae and Pseudomonadaceae are normal habitants of the digestive tract that invade the bloodstream of patients who receive immunosuppressive therapy or suffer from serious underlying trauma or disease, such as severe thermal burns or other serious injuries, cystic fibrosis, renal insufficiency, malignant neoplastic diseases, major surgical procedures, or organ transplantations.

The classic septic shock syndrome results primarily from the sequence of events triggered by bacteremia during which cell wall bacterial substances (endotoxin in Gram-negative organisms and the peptidoglycan/teichoic acid complex in Gram-positive organisms) activate the complement, coagulation, kinin, and ACTH/endorphin systems. This activation results in a series of metabolic events that ultimately progress to a state of shock.

Incipient septic shock is characterized by the following: Body temperature extremes (fever  $>40.6^{\circ}\text{C}$  or hypothermia), altered mental status, orthostatic blood pressure decrease ( $>30$  mm Hg), decreasing urine output, unexplained edema usually associated with a falling serum albumin concentration, tachypnea with hypoxemia, and/or the development of a metabolic acidosis, elevated serum lactate, leukopenia (predominantly neutropenia), and thrombocytopenia with or without petechial skin rash.

Septic shock related to bacteremic infections advances in two hemodynamic stages. First, patients demonstrate symptoms characteristic of vasomotor effect following ACTH/endorphin release, kallikrein-kinin system activation, and histamine release induced by bacterial cell wall components or toxins. As these symptoms develop, complement-mediated leukoagglutination and capillary damage (primary due to the intracapillary adherence and aggregation of activated polymorphonuclear leukocytes) cause a severe capillary leak syndrome followed by a dramatic fall in intravascular blood volume, decline in cardiac output, and disseminated intravascular coagulation.

Obviously, early diagnosis and therapy of patients at risk from septic shock is desirable. At the present time, prophylactic measures include strict adherence to infection control measures, antibiotic and intravenous fluid therapy, immunoprophylaxis, and granulocyte transfusions.

Anti-shock therapy commonly includes volume replacement using plasma expanders such as 5% albumin, isotonic saline, or lactated Ringer's solution under continuous hemodynamic monitoring. Just enough fluid is supplied to bring the patient's pulmonary capillary wedge pressure to the high normal range. Vasoactive compounds such as dopamine, dobutamine, norepinephrine, etc. are used when volume replacement is not sufficient. In addition, anti-inflammatory drugs such as methylprednisolone sodium succinate may be used in large doses (up to 30 mg/kg). Anti-prostaglandins have been proposed for the suppression of inflammatory damage caused by the activated peripheral polymorphonuclear leukocytes.

Finally, mortality from bacteremic infections has been reduced by using substances such as mafenide acetate or silver salts that inhibit bacterial colonization of the burn wound surface and by using potent antimicrobial agents, sympathomimetic amines, corticosteroids, anti-coagulants, granulocyte transfusion, and diuretics for treating bacteremia as primary or adjunct therapy.

Such measures, however, have only proved partially successful in controlling the morbidity and mortality associated with endotoxin or septic shock. Antibiotic therapy may possibly exacerbate incipient toxic shock by inducing the release of bacteria cell wall material and toxins. Vasopressors do not ameliorate capillary damage, anti-inflammatory therapies are controversial, and volume replacement is at best a stop-gap therapy resulting in edema and cardiac complications.

The transforming growth factor- $\beta$  molecules identified thus far are two-chain molecules containing two identical 112 residue polypeptide chains linked by disulfide bonds. The molecular mass of these dimers is about 25 kd. Biologically active TGF- $\beta$  has been defined as a molecule capable of inducing anchorage independent growth of target cell lines or rat fibroblasts in in vitro cell culture, when added together with EGF or TGF- $\alpha$  as a co-factor. Suitable methods are known for purifying TGF- $\beta$  from platelets or placenta, for producing it in recombinant cell culture and for determining its activity. See, for example, R. Derynck et al., *Nature*, 316:701 (1985); EP-A-128,849 PUBLISHED DECEMBER 19, 1984, EP-A-200,341 published December 10, 1986, EP-A-169,016 published January 22, 1986, EP-A-268,561 published May 25, 1988, and EP-A-267,463 published May 18, 1988; GB Pat. Appln. 2,146,335 published April 17, 1985; U.S. Pat. No. 4,774,322; Seyedin et al, *J. Biol. Chem.*, 262: 1946-1949 (1987); and Cheifetz et al, *Cell*, 48: 409-415 (1987).

TGF- $\beta$  has been shown to have numerous regulatory actions on a wide variety of both normal and neoplastic cells. Recent studies indicate an important role for TGF- $\beta$  in cells of the immune system (J. Kehrl et al., *J. Exp. Med.*, 163:1037 [1986]; H-J. Ristow, *Proc. Natl. Acad. Sci. U.S.A.*, 83:5531 [1986]; and A. Rook et al., *J. Immunol.*, 136:3916[1986]) and connective tissue (M. Sporn et al., *Science*, 219:1329 [1983]; R. Ignatz et al., *J. Biol. Chem.*, 261:4337 [1986]; J. Varga et al., *B.B.Res.Comm.*, 138:974 [1986]; A. Roberts et al., *Proc. Natl. Acad. Sci. U.S.A.*, 78:5339 [1981]; A. Roberts et al., *Fed. Proc.*, 42:2621 [1983]; and A. Roberts et al., *Proc. Natl. Acad. Sci. U.S.A.*, 83:4167 [1986]), as well as epithelia (T. Matsui et al., *Proc. Natl. Acad. Sci. U.S.A.*, 83:2438 [1986] and G. Shipley et al. *Cancer Res.*, 46:2068 [1986]). Moreover, TGF- $\beta$  has been described as a suppressor of cytokine (e.g., TNF- $\alpha$ ) production (Espevik et al., *J. Exp. Med.*, 166: 571-576 [1987]) and as a promoter of cachexia (Beutler and Cerami, *New Eng. J. Med.*, 316: 379ff [1987]).

TGF- $\beta$  is multifunctional, since it can either stimulate or inhibit cell proliferation, can either stimulate or inhibit differentiation, and can either stimulate or inhibit other critical processes in cell function (M. Sporn, *Science*, 233:532 [1986]).

The multifunctional activity of TGF- $\beta$  is modulated by the influence of other growth factors present together with the TGF- $\beta$ . TGF- $\beta$  can function as either an inhibitor or an enhancer of anchorage-independent growth, depending on the particular set of growth factors, e.g., EGF or TGF- $\alpha$ , operant in the cell together with TGF- $\beta$  (Roberts et al., *Proc. Natl. Acad. Sci. U.S.A.*, 82:119 [1985]). According to Brinkerhoff et al., *Arthritis and Rheumatism*, 26:1370 (1983), TGF- $\beta$  can act in concert with EGF to cause proliferation and piling up of normal (but not rheumatoid) synovial cells. Furthermore, Chua et al., *J. Biol. Chem.*, 260:5213-5216 [1983] reported that TGF- $\beta$  induced collagenase secretion in human fibroblast cultures, and A. Tashjian et al., *Proc. Natl. Acad. Sci. U.S.A.*, 82:4535 [1985] observed that TGF- $\beta$  stimulated the release of prostaglandins and mobilization of calcium. TGF- $\beta$  also has been reported to inhibit endothelial regeneration (R. Heimark et al., *Science*, 233:1078 [1986]).

PCT W084/01106 published March 29, 1984, relates to the use of TGF- $\beta$  to repair tissue in animals, in particular for use in accelerating wound healing by stimulating cell proliferation. In addition, Sporn et al., *Science*, 219: 1329-1331 (1983) and U.S. Pat. Nos. 4,810,691 and 4,774,228 describe the use of TGF- $\beta$  for promoting connective tissue deposition.

EP-A-269,408, published June 1, 1988, and EP-A-213,776, corresponding to U.S. Pat. No. 4,806,523, disclose use of TGF- $\beta$  as an immunosuppressant, to treat inflammatory diseases such as rheumatoid arthritis.

It has been found that septic shock and invasive infection are diseases caused by humoral mediators of both exogenous and endogenous origin. Thus, release of tumor necrosis factor (TNF), followed by interleukin-1 (IL-1) and interferon-gamma (IFN- $\gamma$ ), participates in the cascade of events noted in Gram-negative sepsis. Hesse et al., *Surg. Gynecol. Obstet.*, 166: 147-153 (1988); Michie et al., *N.Eng.J.Med.*, 318: 1481-1486 (1988); Espevik et al., *J. Immunol.*, 140: 2312-2316 (1988).

Antibodies to TNF were found to protect mice from the lethal effect of endotoxin (Beutler et al., *Science*, 229: 869-871 (1985)). In addition, anti-cachectin/TNF monoclonal antibodies administered two hours before bacterial infusion conferred protection against septic shock and death in baboons. Tracey et al., *Nature*, 330:662-664 (1987). However, it has also been suggested that TNF and IL-1 participate in the mediation of endotoxin-induced enhancement of nonspecific resistance to intraabdominal infection and radiation sickness. Urbaschek et al., *Rev. Infect. Dis.*, 9: S607-S615 (1987).

Monoclonal antibodies directed against endotoxin or its components have been evaluated for their utility in immunotherapy of Gram-negative sepsis. Thus, for example, anti-core lipopolysaccharide (*E. coli*) has been reported to reduce mortality significantly in severely septic patients (E. Zeigler et al., *N. Eng. J. Med.*, 307: 1225 (1982)). Also, murine and human monoclonal antibodies directed against the core lipopolysaccharide of the endotoxin were found to exert protection during Gram-negative bacterial sepsis in animals.

Dunn, Transplantation, 45: 424-429 (1988); EP-A-183,876 published June 11, 1986; EP-A-174,204 published March 12, 1986. Antibodies directed against lipid A also had a protective effect in humans. Jaspers et al., Infection, 15 Suppl. 2: S89-95 (1987). Antibodies to the J5 mutant of E. coli are reported to be protective against septic shock in animals and humans. Cohen et al., Lancet, 1:8-11 (1987); Law and Marks, J. Infect. Dis., 151: 988-994 (1985). Antibodies to endotoxin core glycolipid were found to prevent the serious consequences of Gram-negative infections in surgical patients. Baumgartner et al., Lancet, 2: 59-63 (1985). In addition, human monoclonal antibodies to P. aeruginosa exotoxin A and exoenzyme S have been described as useful for this purpose. U.S. Pat. No. 4,677,070 issued June 30, 1987 and EP-A-243,174 published Oct. 28, 1987, respectively.

The clinical utility of these approaches using antibodies is being evaluated, but it is believed that they may suffer from some disadvantages such as unfavorable kinetics, biological half-life, and the potential for anti-idiotypic antibody generation that would neutralize the therapeutic antibody (in the case of human antibodies). In addition, this immunotherapy only interdicts an early-stage effector.

It is an object of this invention to provide methods and compositions for the effective therapy and prevention of septic shock that do not rely on monoclonal antibody therapy.

This and other objects will become apparent to one of ordinary skill in the art.

These objects are achieved, in one aspect, by the use of TGF- $\beta$  in the manufacture of a medicament for the treatment of a patient suffering from or at risk of septic shock.

In another aspect, this invention provides a composition for the treatment or prevention of septic shock comprising a pharmaceutically effective amount of transforming growth factor-beta (TGF- $\beta$ ) and a pharmaceutically effective amount of a substance selected from mafenide acetate, an anti-microbial agent, a sympathomimetic amine, a corticosteroid, an anti-coagulant, a diuretic, an antibody against lipid A, an antibody against endotoxin core glycolipid, an antibody against J5 mutant of E. coli, and a mixture of two or more of these substances.

According to a further aspect the invention provides for use of TGF- $\beta$  and at least one of mafenide acetate, an anti-microbial agent, a sympathomimetic amine, a corticosteroid, an anti-inflammatory agent, an anti-coagulant, a diuretic, an antibody against tumour necrosis factor, an antibody against lipid A, an antibody against endotoxin core glycolipid, an antibody against J5 mutant of E. coli, an antagonist to tumour necrosis factor, (or) an antagonist to interleukin-1 in the manufacture of a medicament for treatment of a patient suffering from or at risk of septic shock.

Figures 1a and 1b show the cDNA sequences and deduced amino acid sequences of porcine and human TGF- $\beta_3$ , respectively. The 112 amino acid sequence of mature TGF- $\beta_3$  (overlined) constitutes the C-terminus of the precursor and is preceded by four basic residues (+). The precursor segment contains four overlined potential N-glycosylation sites. All cysteine residues are shaded. The AATAAA (Fig. 1a) and the related AGTAAA (Fig. 1b) sequence close to the 3' end of the cDNA and preceding the polyadenylation site are underlined. Fig. 1c shows the homology between the imputed amino acid sequences of the human (h) and porcine (p) TGF- $\beta_3$  precursors. The asterisks mark identical residues, while a dot indicates a conservative amino acid replacement. The mature TGF- $\beta_3$  sequences are boxed.

Figure 2 shows the N-termini for selected forms of TGF- $\beta$ . The letters h, p and b stand for human, porcine and bovine, respectively. Non-homologous residues are designated by a period over the residue in question.

Figure 3 shows the percent cumulative lethality of mice over time upon treatment with recombinant human TGF- $\beta$  or recombinant human IL-1 $\alpha$  versus the PBS control at various doses and times relative to challenge with endotoxin.

Figure 4 shows a separate study from Fig. 3 on the percent cumulative lethality of mice over time upon treatment with recombinant human TGF- $\beta$  versus the PBS control at various doses and times relative to challenge with endotoxin.

Figure 5 shows the percent cumulative lethality upon treatment of mice with recombinant human TGF- $\beta$  or recombinant human IL-1 $\alpha$  versus the PBS control after cecal ligation and puncture surgery.

As used herein, the expression "septic shock" refers to a condition primarily caused by bacteremic Gram-negative infection of a patient. This pathologic condition, of which endotoxin shock is a subset, is characterized by such symptoms as irreversible cardiovascular collapse and critical organ failure. In addition, the patient ordinarily experiences hyperventilation, skin lesions, and hypotension, and in certain instances, fever, chills, fall in urine output, and decrease in circulating platelet levels, jaundice, acidosis, bleeding, and hypoxia. Examples of Gram-negative bacteria responsible for septic shock include E. coli, including, e.g., the J5 mutant of E. coli (having only core determinants in its endotoxin, analogous to an Rc mutant), Pseudomonas aeruginosa, Aeromonas hydrophila, Yersinia Pestis, and species from Klebsiella-Enterobacter, Salmonella, Hemophilus, Proteus, and Serratia. Each species may produce different symp-

toms in the patient.

At the present time five highly homologous forms of TGF- $\beta$  have been identified, TGF- $\beta_1$ , TGF- $\beta_2$ , TGF- $\beta_3$ , TGF- $\beta_4$ , and TGF- $\beta_5$ . N-termini for the first three of these forms are set forth in Fig. 2. Reference to TGF- $\beta$  herein will be understood as reference to any one of these identified forms as well as others identified in the future, their alleles, and their predetermined amino acid sequence variants, so long as they are effective in the method described herein.

As can be seen from Figs. 1a-1c, the mature TGF- $\beta_3$  amino acid sequence contains a large number of cysteine residues, at least some of which apparently are involved in interchain crosslinking in forming the homodimeric TGF- $\beta$  that is recovered from natural sources. The rest of the precursor contains only two cysteine residues. The complete TGF- $\beta_3$  precursor contains several pairs of basic residues that could also undergo post-translation cleavage and give rise to separate polypeptide entities.

Comparison of the porcine and human TGF- $\beta_3$  precursor sequences (see Fig. 1c) reveals a 90% amino acid identity. The amino acid sequences predicted from the human and porcine cDNA sequences are 410 and 409 amino acids long, respectively, and have a C-terminal sequence that resembles the previously established sequences for mature TGF- $\beta$ . The C-terminal 112 amino acid sequence has about 80% similarity to the porcine and human TGF- $\beta_1$  sequence and shares a similar degree of homology with the sequence of TGF- $\beta_2$ .

In the treatment or prophylaxis of septic shock the TGF- $\beta$  is administered prophylactically or therapeutically, i.e., before, simultaneous with, or after the infection has set in. The TGF- $\beta$  may be used passively to treat individuals who suffer from septicemia or are at risk with respect to bacteremic Gram-negative infection. Patients at risk include those receiving immunosuppressive therapy and those suffering from severe thermal burns or other serious injuries, cystic fibrosis, renal failure, or cancer, or are undergoing extensive surgical procedures or organ transplantation. One possible treatment is for chronic endobronchitic infection endemic in cystic fibrosis patients.

It is within the scope hereof to employ TGF- $\beta$  from animals other than humans, for example, porcine or bovine sources, to treat humans. Likewise, if it is desirable to treat other mammalian species such as domestic and farm animals and sports or pet animals, human TGF- $\beta$ , as well as TGF- $\beta$  from other species, is suitably employed. In one instance of animal treatment, dairy cows are treated for acute coliform mastitis infections by using TGF- $\beta$  to remove and neutralize the effects of endotoxin. Thus, the term "patient" as used herein refers to all mammals, not just humans.

The TGF- $\beta$  is administered to the patient by any suitable technique, including parenteral and, if desired for localized bacteria, intralesional administration, preferably parenteral administration. The specific method of administration will depend, e.g., on whether the administration is therapeutic or prophylactic. Thus, in view of the therapeutic urgency attendant frank shock, the TGF- $\beta$  is preferably intravenously infused at the same time as solutions used for initial volume expansion. However, prophylaxis is generally accomplished, e.g., by intramuscular or subcutaneous administration or other parenteral administration, including intraarterial and intraperitoneal administration, preferably intravenous or intraperitoneal.

The TGF- $\beta$  compositions to be used in the therapy will be formulated and dosed in a fashion consistent with good medical practice taking into account the clinical condition of the individual patient, the cause of the septic shock, whether the TGF- $\beta$  is used for therapy of frank shock or prophylaxis of incipient septic shock, the site of delivery of the TGF- $\beta$ , the method of administration, the scheduling of administration, and other factors known to practitioners. The "effective amount" for purposes herein is thus determined by such considerations.

As a general proposition, the total pharmaceutically effective amount of the TGF- $\beta$  administered parenterally per dose will be in the range of approximately 1  $\mu$ g/kg to 1 mg/kg of patient body weight once per day, although, as noted above, this will be subject to a great deal of therapeutic discretion. The key factor in selecting an appropriate dose and scheduling is the result obtained. Relatively higher doses may be needed initially for the treatment of profound shock, i.e., for patients in acute renal failure or respiratory distress, or having severely depressed blood pressure (mean arterial pressure below about 60 mm Hg).

The TGF- $\beta$  is used in an activated form as well as in latent forms for slow-release formulations. Preferably, the TGF- $\beta$  is activated, as by such methods as exposure to acidic or basic pH values, sodium dodecyl sulfate, or high concentrations of urea, as described, e.g., in Miyazono et al. *J. Biol. Chem.*, 263: 6407-6415 (1988). For example, the TGF- $\beta$  may be treated with acid to give activity at pH below 6, preferably below 5.5, or incubated with 0.02% SDS or 8 M urea.

For parenteral administration, the TGF- $\beta$  is formulated generally by mixing it at the desired degree of purity, in a unit dosage injectable form (solution, suspension, or emulsion), with a physiologically acceptable carrier, i.e., one that is non-toxic to recipients at the dosages and concentrations employed. Preferably the carrier is a parenteral carrier. Examples of such carrier vehicles include water, saline, Ringer's solution,

dextrose solution, and 5% human serum albumin. Non-aqueous vehicles such as fixed oils and ethyl oleate are also useful herein, as well as liposomes. Generally, the carrier can contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, e.g., buffers and preservatives, as well as low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose or dextrans, chelating agents such as EDTA, or other excipients. The TGF- $\beta$  is typically formulated in such vehicles at a concentration of about 0.1 mg/ml to 100 mg/ml at pH range 4 to 6.

TGF- $\beta$  for use in therapeutic administration must be sterile. This is readily accomplished by sterile filtration through (0.2 micron) membranes. TGF- $\beta$  ordinarily will be stored as an aqueous solution since it is highly stable to thermal and oxidative denaturation, although lyophilized formulations for reconstitution are acceptable.

TGF- $\beta$  therapy or prophylaxis is suitably combined with other proposed or conventional therapies or prophylactic treatment for septic shock. For example, for treatment of burns, the TGF- $\beta$  therapy may be delivered by separate means, simultaneously with and by the same administration route as other substances such as mafenide acetate, antibiotics, or anti-microbial agents that inhibit bacterial colonization of the burn wound surface. Other therapies that can be combined with TGF- $\beta$  therapy include primary therapeutic agents, for example, potent anti-microbial agents such as aminoglycosides (such as amikacin, tobramycin, netilmicin, and gentamicin), cephalosporin, related beta-lactam agents such as moxalactam, carbopenems such as imipenem, monobactam agents such as aztreonam, ampicillin, and broad-spectrum penicillins (eg., penicillinase-resistant penicillins, ureidopenicillins, or antipseudomonal penicillins) that are active against *P. aeruginosa*, *Enterobacter* species, indole-positive *Proteus* species, and *Serratia*.

Various adjunctive agents in the treatment of septic shock also are useful in combination with TGF- $\beta$ . These include sympathomimetic amines (vasopressors) such as norepinephrine, epinephrine, isoproterenol, dopamine, and dobutamine; anti-inflammatory agents such as methylprednisolone, corticosteroids such as betamethasone, hydrocortisone, methylprednisolone, or dexamethasone; anti-coagulants such as heparin or coumadine-type drugs for certain conditions and schedules; diuretics such as furosemide or ethacrynic acid; an antagonist of opiates and beta-endorphins such as naloxone; an antagonist of tumor necrosis factor or of interleukin-1; phenothiazines; anti-histamines; anti-inflammatory agents such as indomethacin and phenylbutozone; glucagon;  $\alpha$ -adrenergic blocking agents, vasodilators; plasma expanders, packed red blood cells; platelets; cryoprecipitates; fresh frozen plasma; clindamycin; antibodies to the J5 mutant of *E. coli*, to lipid A, or to endotoxin core glycolipids. Methods for preparing the antibodies are described in the articles provided supra.

Therapeutic measures that can be used in conjunction with administering the TGF- $\beta$  include granulocyte transfusion and percutaneous drainage of abdominal abscesses. In addition, prophylactic measures that are useful in conjunction with the TGF- $\beta$  involve, e.g., barrier isolation to minimize contact of the patient with infectious agents, use of prophylactic anti-microbial agents of a systemic form, active or passive immunoprophylaxis with type-specific or cross-reactive antibodies, and augmentation of the host granulocyte pool with prophylactic granulocyte transfusions.

It is not necessary that such cotreatment drugs be included in the TGF- $\beta$  compositions per se, although this will be convenient where such drugs are delivered by the same administration route, e.g., antagonists to the activity of TNF- $\alpha$  or IL-1, or the above-described neutralizing antibodies.

When employed together with the TGF-beta, such agents (other than antibiotics) preferably are employed in lesser dosages than when used alone. A typical combined composition will contain greater than about 0.5 nmole, generally about 0.05  $\mu$ mole, of TGF-beta, about from 0.0003 to 0.05  $\mu$ mole of anti-lipopolysaccharide IgG, and about from 5 to 60  $\mu$ g of dopamine in about from 10 to 1000 ml of a suitable intravenous or intraperitoneal fluid such as lactated Ringer's solution. This composition then is piggybacked onto an infusion serving primarily for plasma expansion and administered to control shock.

The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention.

#### EXAMPLE 1

The experiments described below were designed to determine the effects of TGF- $\beta$  on LPS-induced TNF- $\alpha$  production *in vivo*.

Five groups of female BALB/c mice, 6-8 weeks old, with six mice per group, were treated as follows:

Group A: LPS (*E. coli* LPS 026:B6, Sigma Chemical Co., St. Louis, MO) at 5  $\mu$ g/mouse was administered iv at 5  $\mu$ l/mouse.

Group B: Phosphate buffered saline was administered at four hours before time 0 (when LPS was administered) and at time 0.

Group C: TGF- $\beta$  at 1  $\mu$ g/mouse was administered iv at 100  $\mu$ l/mouse at four hours prior to administration of LPS (at time 0) at 5  $\mu$ g/mouse iv at 100  $\mu$ l/mouse. The TGF- $\beta$  employed in this experiment was human TGF- $\beta$ 1 obtained recombinantly as described in EP-A-200,341 published Dec. 10, 1986. The diluent employed for the TGF- $\beta$  was 20 mM sodium acetate buffer, pH 4.

Three mice per group were sacrificed and bled 60 minutes after LPS treatment and the other three mice per group were sacrificed and bled 90 minutes after LPS treatment. The sera from the mice were harvested and stored at -70 °C. Then the amount of TNF- $\alpha$  in the sera was measured by subjecting the sera to the MTT tetrazolium cytotoxicity assay using WEHI 164 clone 13 mouse fibrosarcoma cells described by Espevik et al., *J.Immunol.Meth.*, 95: 99-105 (1986). The results are shown in the table below:

Test No.	Agent	TNF- $\alpha$ (pg/ml)	Average TNF- $\alpha$ (pg/ml) per every three experiments
1	LPS	117 +/- 21	273 +/- 85
2 60'	LPS	410 +/- 33	
3	LPS	293 +/- 40	
4	LPS	525 +/- 4	369 +/- 78
5 90'	LPS	312 +/- 0	
6	LPS	270 +/- 10	
7	PBS	<0.2	
8 60'	PBS	<0.2	
9	PBS	<0.2	
10	PBS	<0.2	
11 90'	PBS	<0.2	
12	PBS	<0.2	236 +/- 97
13	1 $\mu$ g TGF- $\beta$	72 +/- 6	
14 60'	1 $\mu$ g TGF- $\beta$	409 +/- 19	
15	1 $\mu$ g TGF- $\beta$	227 +/- 14	
16	1 $\mu$ g TGF- $\beta$	97 +/- 8	
17 90'	1 $\mu$ g TGF- $\beta$	203 +/- 17	241 +/- 97
18	1 $\mu$ g TGF- $\beta$	423 +/- 47	
19	5 $\mu$ g TGF- $\beta$	443 +/- 30	
20 60'	5 $\mu$ g TGF- $\beta$	389 +/- 20	649 +/- 233
21	5 $\mu$ g TGF- $\beta$	1114 +/- 72	
22	5 $\mu$ g TGF- $\beta$	601 +/- 2	
23 90'	5 $\mu$ g TGF- $\beta$	379 +/- 40	561 +/- 324
24	5 $\mu$ g TGF- $\beta$	705 +/- 68	
25	10 $\mu$ g TGF- $\beta$	1039 +/- 64	
26 60'	10 $\mu$ g TGF- $\beta$	401 +/- 76	791 +/- 197
27	10 $\mu$ g TGF- $\beta$	935 +/- 67	
28	10 $\mu$ g TGF- $\beta$	2292 +/- 24	
29 90'	10 $\mu$ g TGF- $\beta$	2118 +/- 83	1842 +/- 365
30	10 $\mu$ g TGF- $\beta$	1118 +/- 13	

The results indicate that TGF- $\beta$  inhibits endotoxin-induced TNF- $\alpha$  production at lower doses; at higher doses it significantly enhances TNF- $\alpha$  production.

A similar experiment using female NMRI mice revealed the following: Whereas no TNF- $\alpha$  was detectable in controls and in mice two hours after 5  $\mu$ g of TGF- $\beta$  was administered ip, 770 U/ml of TNF- $\alpha$  were detected two hours after 10  $\mu$ g of endotoxin was administered and 240 U/ml of TNF- $\alpha$  were detected when TGF- $\beta$  (5  $\mu$ g) was injected simultaneously with 10  $\mu$ g of endotoxin.

## EXAMPLE 2

### First Endotoxin Tolerance Model

Five groups of NMRI female mice (weighing 28.2 +/- 3.4 g) and 9-10 weeks old) were used, each group consisting of ten animals. The mice were permitted food ad libitum. One group received 5  $\mu$ g of TGF- $\beta$  at -24 hours, the second at the time of endotoxin administration, the third at -24 hours and again at the time of endotoxin administration, and the fourth received 2000 units of recombinant human IL-1 $\alpha$  at -24

hours. The fifth group was the control, which consisted of mice treated with phosphate buffered saline at 0.5 ml at -24 hours. The administration of TGF- $\beta$  and IL-1 was intraperitoneal, and the administration of the endotoxin was intravenous at 200  $\mu$ g or 0.2 ml per injection.

The TGF- $\beta$  employed in this experiment was the same as that used in Example 1. After purification, the TGF- $\beta$  was formulated in acetic acid buffer at pH 5 to 5.4. The IL-1 was recombinant human IL-1 $\alpha$  obtained from Dr. Lomedico at Roche Pharmaceuticals, Nutley, N.J. (As IL-1 $\alpha$  and IL-1 $\beta$  bind to the same receptor, similar results are expected using IL-1 $\beta$ .) The endotoxin was extracted from *E. coli* 0111:B4 (publicly available) by the method described by Boivin et al., *C.R. Soc. Biol.*, 114: 307-310 (1933), Boivin et al., *C.R. Soc. Biol.*, 115: 304 (1934), and Boivin et al., *Rev.d'Immunologie*, 1: 553 (1935).

The percent mortality of the mice was determined after one, two, three, and four days. The results of this study are shown in Table I, and the plots of the percent cumulative lethality of each group of mice versus days is shown in Figure 3, where the crosses are the TGF- $\beta$  pretreatment, the closed circles are the TGF- $\beta$  pretreatment/simultaneous treatment, the closed triangles are the TGF- $\beta$  simultaneous treatment, the dashed line is the IL-1 treatment, and the asterisks are the PBS control.

TABLE I

Agent	Dose	Time	Percent Cumulative Lethality at Day 4 After Challenge
TGF- $\beta$	5 $\mu$ g	-24 hr.	30
TGF- $\beta$	5 $\mu$ g/5 $\mu$ g	-24/0 hr.	40
TGF- $\beta$	5 $\mu$ g	0 hr.	50
IL-1	2000 units	-24 hr.	70
PBS (control)	0.5 ml	-24 hr	70

Percent mortality was reduced in all cases over the control when TGF- $\beta$  was employed. The percent mortality was the lowest when the TGF- $\beta$  was administered once at -24 hours.

#### Second Endotoxin Tolerance Model

The above experiment was repeated using a 190  $\mu$ g/0.2 ml iv dose of the endotoxin, groups of ten mice, and varying ip doses of the TGF- $\beta$  used above (i.e., 5  $\mu$ g/0.25 ml at 24 hours before endotoxin administration, 5  $\mu$ g/0.25 ml simultaneously with endotoxin administration, and 10  $\mu$ g/0.25 ml simultaneously with endotoxin administration). The percent cumulative lethality profiles from 1 to 4 days after endotoxin challenge are shown in Fig. 4, where the open squares are the control, the crosses are the TGF- $\beta$  24 hours before endotoxin administration, the open circles are 5  $\mu$ g TGF- $\beta$  simultaneously with endotoxin administration, and the closed circles are 10  $\mu$ g TGF- $\beta$  simultaneously with endotoxin administration. The 10  $\mu$ g/mouse dose of TGF- $\beta$  was found to enhance mortality after endotoxin administration.

#### Septicemia/Cecal Ligation Model

Because of the potential immunosuppressive effects of TGF- $\beta$ , a study was performed to see if TGF- $\beta$  would increase the susceptibility of mice to bacterial infection due to a puncture wound.

TGF- $\beta$  and IL-1 were administered separately, intraperitoneally, at 5  $\mu$ g dose of TGF- $\beta$  and at 2000 units dose of IL-1 once at 24 hours prior to surgery on the mice, performed as described below. A control was employed consisting of PBS administered intraperitoneally at 0.5 ml.

Experiments were performed in groups of seven female NMRI mice (weighing 28.2  $\pm$  3.4 g), which were permitted food ad libitum. A 0.5-cm midline incision was made in etherized animals, and the cecums were carefully exteriorized. Each cecum was filled with feces by milking stool into it from the ascending colon and then ligated just below the ileocecal valve to retain bowel continuity. After two punctures of the cecum with a 20-gauge needle followed by slight pressure to force the appearance of feces, the cecum was returned to the peritoneal cavity and the incision closed with a clamp. Finally, the operated mice received 1 ml of saline subcutaneously.

The mice were then evaluated for percent lethality every day for eight days following the surgery. The results are shown in Figure 5. The open circles represent the control, the closed circles represent the IL-1, and the crosses represent TGF- $\beta$ . IL-1 was found to reduce mortality relative to the control, whereas TGF- $\beta$  did not decrease or substantially increase mortality relative to the control. Thus, TGF- $\beta$ , which is a known



immunosuppressive agent, did not increase the mortality of the mice.

### EXAMPLE 3

#### 5 Effects on *E. coli* Septic Shock

Recent evidence suggests that tumor necrosis factor-alpha is a key mediator of the lethal effects of overwhelming bacterial infection. The administration of antibodies to TNF- $\alpha$ , in contrast, protects animals from the lethal effects of septic shock. As TGF- $\beta$  can decrease endotoxin-stimulated release of TNF- $\alpha$  *in vitro* and *in vivo* (J. Exp. Med., 166: 571 (1987)), the ability of TGF- $\beta$  to inhibit *E. coli*-induced septic shock was investigated, as described below:

Fischer 344 male rats of 180-200 g were administered intraperitoneally 10  $\mu$ g of either recombinant TGF- $\beta$ 1 (prepared as described in Example 1 in a buffer comprising 20 mM sodium acetate pH 5 with 0.1% weight:volume human serum albumin), recombinant TGF- $\beta$ 2 (obtained from Dr. Adriano Fontana, Zurich, Switzerland, as a lyophilized pellet--see WO 88/03807 published June 2, 1988--and resuspended in 1% acetic acid), or diluent control (consisting of a buffer of 20 mM sodium acetate with 0.1% human serum albumin). 72 hours later, pathogenic *E. coli* ( $1-3 \times 10^9$ ) was given. The results are indicated below:

Substance	Mortality (dead/total) hours after <i>E. coli</i> Challenge			
	12	24	48	72
Control	5/30	8/30	17/30	18/30
rTGF- $\beta$ 1	0/30	7/30	12/30	13/30
Control	2/12	6/12	11/12	11/12
rTGF- $\beta$ 2	0/12	4/12	5/12	5/12

It was found that 1-25  $\mu$ g of TGF- $\beta$ 1 in this rat model was effective in reducing mortality versus the diluent control; however, a dose of 50  $\mu$ g of TGF- $\beta$ 1 increased mortality versus the control. This is consistent with the dose-dependent results from the experiments reported in Example 1.

#### Serum TNF- $\alpha$ Levels

In parallel experiments reduced serum TNF- $\alpha$  (and IL-6) levels were determined two hours after *E. coli* injection by the L-M *in vitro* bioassay described by Kramer et al., J. Immunol. Meth., 93: 201-206 (1986). The serum TNF- $\alpha$  levels were found to be 22  $\pm$  4.9 units/ml (5 rats) versus 87.5  $\pm$  18.6 units/ml (5 rats) for controls,  $p < 0.01$ .

These data suggest that *in vivo* rTGF- $\beta$  pretreatment can reduce the peak TNF- $\alpha$  response to *E. coli* challenge as well as delay and reduce the mortality observed with septic shock.

#### Claims

1. Use of transforming growth factor -beta (TGF- $\beta$ ) in the manufacture of a medicament for treatment of a patient suffering from or at risk of septic shock.
2. A use according to claim 1 wherein the TGF- $\beta$  is TGF- $\beta$ 1, TGF- $\beta$ 2, or TGF- $\beta$ 3.
3. A use according to claim 1 or claim 2 wherein the TGF- $\beta$  is human TGF- $\beta$ .
4. A use according to any one of the preceding claims wherein the medicament is adapted for administration of about 5  $\mu$ g to 1 mg of TGF- $\beta$  per kg of patient body weight and is for administration parenterally on a daily basis.
5. A use according to any one of the preceding claims wherein the medicament is for treatment of a patient having a microbial infection but not yet showing symptoms of septic shock.
6. A use according to any one of the preceding claims wherein the TGF- $\beta$  is for administration by intravenous infusion or intraperitoneally.

7. A composition for the treatment or prevention of septic shock comprising a pharmaceutically effective amount of transforming growth factor-beta (TGF- $\beta$ ) and a pharmaceutically effective amount of at least one of mafenide acetate, an anti-microbial agent, a sympathomimetic amine, a corticosteroid, an anti-coagulant, a diuretic, an antibody against lipid A, an antibody against endotoxin core glycolipid, or an antibody against J5 mutant of E.coli.  
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8. The composition of claim 7 wherein the composition is formulated in a pharmaceutically acceptable carrier.
- 10 9. The composition of claim 8 wherein the carrier is parenteral.
10. The composition of any one of claims 7-9 wherein the TGF- $\beta$  is TGF- $\beta_1$ , TGF- $\beta_2$ , or TGF- $\beta_3$ .
11. The composition of claim 10 wherein the TGF- $\beta$  is human TGF- $\beta$ .
- 15 12. The composition of any one of claims 7-11 that is isotonic.
13. A pharmaceutical product comprising a pharmaceutically effective amount of TGF- $\beta$  and a pharmaceutically effective amount of at least one of mafenide acetate, an anti-microbial agent, a sympathomimetic amine, a corticosteroid, an anti-coagulant, a diuretic, an antibody against lipid A, an antibody against endotoxin core glycolipid, or an antibody against J5 mutant of E.coli as a combined preparation for simultaneous, separate or sequential use in treatment of a patient suffering from or at risk of septic shock.  
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- 25 14. Use of TGF- $\beta$  and at least one of mafenide acetate, an anti-microbial agent, a sympathomimetic amine, a corticosteroid, an anti-inflammatory agent, an anti-coagulant, a diuretic, an antibody against tumour necrosis factor, an antibody against lipid A, an antibody against endotoxin core glycolipid, an antibody against J5 mutant of E.coli, an antagonist to tumour necrosis factor, or an antagonist to interleukin-1 in the manufacture of a medicament for treatment of a patient suffering from or at risk of septic shock.  
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#### Patentansprüche

1. Verwendung von transformierendem Wachstumsfaktor-beta (TGF- $\beta$ ) bei der Herstellung eines Medikaments zur Behandlung eines Patienten, der an septischem Schock leidet oder davon bedroht ist.  
35
2. Verwendung nach Anspruch 1, worin der TGF- $\beta$  TGF- $\beta_1$ , TGF- $\beta_2$  oder TGF- $\beta_3$  ist.
3. Verwendung nach Anspruch 1 oder 2, worin der TGF- $\beta$  menschlicher TGF- $\beta$  ist.
- 40 4. Verwendung nach einem der vorhergehenden Ansprüche, worin das Medikament zur Verabreichung von etwa 5  $\mu$ g bis 1 mg TGF- $\beta$  pro kg Körpergewicht des Patienten angepaßt und zur parenteralen Verabreichung auf einer täglichen Basis bestimmt ist.
5. Verwendung nach einem der vorhergehenden Ansprüche, worin das Medikament zur Behandlung eines Patienten bestimmt ist, der eine mikrobielle Infektion aufweist, aber noch keine Symptome von septischem Schock zeigt.  
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6. Verwendung nach einem der vorhergehenden Ansprüche, worin der TGF- $\beta$  zur Verabreichung durch intravenöse Infusion oder intraperitoneal bestimmt ist.
- 50 7. Zusammensetzung zur Behandlung oder Prävention von septischem Schock, die eine pharmazeutisch wirksame Menge transformierenden Wachstumsfaktor-beta (TGF- $\beta$ ) und eine pharmazeutisch wirksame Menge an zumindest einem Mitglied der Gruppe enthaltend Mafenidacetat, ein antimikrobielles Mittel, ein sympathomimetisches Amin, ein Corticosteroid, einen Koagulationshemmer, ein Diuretikum, einen Antikörper gegen Lipid A, einen Antikörper gegen Endotoxinernglykolipid und einen Antikörper gegen J5 Mutant von E.coli, umfaßt.  
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8. Zusammensetzung nach Anspruch 7, worin die Zusammensetzung in einem pharmazeutisch annehmbaren Träger formuliert ist.
9. Zusammensetzung nach Anspruch 8, worin der Träger parenteral ist.
10. Zusammensetzung nach einem der Ansprüche 7 bis 9, worin der TGF- $\beta$  TGF- $\beta_1$ , TGF- $\beta_2$  oder TGF- $\beta_3$  ist.
11. Zusammensetzung nach Anspruch 10, worin der TGF- $\beta$  menschlicher TGF- $\beta$  ist.
12. Zusammensetzung nach einem der Ansprüche 7 bis 11, die isotonisch ist.
13. Pharmazeutisches Produkt, das eine pharmazeutisch wirksame Menge TGF- $\beta$  und eine pharmazeutisch wirksame Menge von zumindest einem Mitglied der Gruppe enthaltend Mafenidacetat, ein antimikrobielles Mittel, ein sympathomimetisches Amin, ein Corticosteroid, einen Koagulationshemmer, ein Diuretikum, einen Antikörper gegen Lipid A, einen Antikörper gegen Endotoxinkernglykolipid und einen Antikörper gegen J5 Mutant von E.coli, umfaßt, als kombiniertes Präparat zur gleichzeitigen, getrennten oder sequentiellen Verwendung bei der Behandlung eines Patienten, der an septischem Schock leidet oder davon bedroht ist.
14. Verwendung von TGF- $\beta$  und zumindest einem Mitglied der Gruppe enthaltend Mafenidacetat, ein antimikrobielles Mittel, ein sympathomimetisches Amin, ein Corticosteroid, ein entzündungshemmendes Mittel, einen Koagulationshemmer, ein Diuretikum, einen Antikörper gegen Tumornekrosefaktor, einen Antikörper gegen Lipid A, einen Antikörper gegen Endotoxinkernglykolipid, einen Antikörper gegen J5 Mutant von E.coli, einen Antagonisten gegen Tumornekrosefaktor und einen Antagonisten gegen Interleukin-1, bei der Herstellung eines Medikaments zur Behandlung eines Patienten, der an septischem Schock leidet oder davon bedroht ist.

#### Revendications

1. Utilisation du facteur de croissance-transformation bêta (TGF- $\beta$ ) dans la production d'un médicament destiné au traitement d'un patient souffrant ou présentant un risque de choc septique.
2. Utilisation suivant la revendication 1, dans laquelle le TGF- $\beta$  est le TGF- $\beta_1$ , le TGF- $\beta_2$  ou le TGF- $\beta_3$ .
3. Utilisation suivant la revendication 1 ou la revendication 2, dans laquelle le TGF- $\beta$  est le TGF- $\beta$  humain.
4. Utilisation suivant l'une quelconque des revendications précédentes, dans laquelle le médicament est apte à l'administration d'environ 5  $\mu$ g à 1 mg de TGF- $\beta$  par kilogramme de poids corporel du patient et est destiné à une administration parentérale journalière.
5. Utilisation suivant l'une quelconque des revendications précédentes, dans laquelle le médicament est destiné au traitement d'un patient présentant une infection microbienne, mais ne présentant pas encore de symptômes de choc septique.
6. Utilisation suivant l'une quelconque des revendications précédentes, dans laquelle le TGF- $\beta$  est destiné à une administration par perfusion intraveineuse ou par voie intrapéritonéale.
7. Composition destinée au traitement ou à la prévention du choc septique, comprenant une quantité pharmaceutiquement efficace du facteur de croissance-transformation bêta (TGF- $\beta$ ) et une quantité pharmaceutiquement efficace d'au moins un composé choisi entre l'acétate de mafénide, un agent antimicrobien, une amine sympathomimétique, un corticostéroïde, un anticoagulant, un diurétique, un anticorps contre le lipide A, un anticorps contre le glycolipide central d'endotoxine et un anticorps contre le mutant J5 de E.coli.
8. Composition suivant la revendication 7, qui est formulée avec un véhicule pharmaceutiquement acceptable.

9. Composition suivant la revendication 8, dans laquelle le véhicule est un véhicule pour administration parentérale.

10. Composition suivant l'une quelconque des revendications 7 à 9, dans laquelle le TGF- $\beta$  est le TGF- $\beta_1$ , le TGF- $\beta_2$  ou le TGF- $\beta_3$ .

11. Composition suivant la revendication 10, dans laquelle le TGF- $\beta$  est le TGF- $\beta$  humain.

12. Composition suivant l'une quelconque des revendications 7 à 11, qui est isotonique.

13. Produit pharmaceutique comprenant une quantité pharmaceutiquement efficace de TGF- $\beta$  et une quantité pharmaceutiquement efficace d'au moins un composé choisi entre l'acétate de mafénide, un agent antimicrobien, une amine sympathomimétique, un corticostéroïde, un anticoagulant, un diurétique, un anticorps contre le lipide A, un anticorps contre le glycolipide central d'endotoxine et un anticorps contre le mutant J5 de E.coli, sous forme d'une préparation en association pour une utilisation de manière simultanée, distincte ou successive dans le traitement d'un patient souffrant ou présentant un risque de choc septique.

14. Utilisation du TGF- $\beta$  et d'au moins un composé choisi entre l'acétate de mafénide, un agent antimicrobien, une amine sympathomimétique, un corticostéroïde, un agent anti-inflammatoire, un anticoagulant, un diurétique, un anticorps contre le facteur de nécrose tumorale, un anticorps contre le lipide A, un anticorps contre le glycolipide central d'endotoxine, un anticorps contre le mutant J5 de E.coli, un antagoniste du facteur de nécrose tumorale et un antagoniste de l'interleukine-1 dans la production d'un médicament destiné au traitement d'un patient souffrant ou présentant un risque de choc septique.

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Fig. 1a.(cont.) I.

180  
 ILE LEU GLN PRO ASP GLN HIS ILE ALA LYS GLN ARG TYR ILE ASP GLY LYS ASN LEU PRO THR ARG GLY ALA ALA GLU TRP  
 778 ATC CTC CAG CCC GAT GAG CAC ATA GCC AAG CAG CGC TAC ATC GAC GGC ARG AAC CTC CCC ACG CGG GGT GCC GCG GAG TGG  
 220  
 LEU SER PHE ASP VAL THR ASP THR VAL ARG GLN TRP LEU LEU ARG ARG GLN SER ASN LEU GLY LEU GLU ILE SER ILE HIS  
 859 CTG TCC TTC GAC GTC ACA GAC ACT GTG CGT GAA TGG CTC TTG AGA AGA GAA TCC AAC TTG GGT CTG GAA ATC AGC ATT CAT  
 230  
 MET PRO LYS HIS THR PHE GLN PRO ASN GLY ASP ILE LEU GLN ASN ILE GLN GLN VAL MET GLU ILE LYS PHE LYS GLY VAL  
 940 TGT CCG TGT CAC ACC TTT CAG CCC AAC GGG GAT ATC TTG GAA AAC ATT CAA GAG GTG ATG GAA ATC AAA TTC AAA GGT GTG  
 240  
 ASP SER GLN ASP PRO GLY ARG GLY ASP LEU LEU ARG LEU LYS LYS LYS GLN HIS SER PRO HIS LEU ILE LEU MET  
 1021 GAC AGT GAG GAT GAT CCG GGC CGT GGA GAC CTG GGG CGA CTT AAG AAG AAG AAG GAA CAC AGC CCT CAT CTA ATC CTC ATG  
 270  
 MET ILE PRO PRO ASP ARG LEU ASP ASN PRO GLY LEU GLY ALA GLN ARG LYS LYS ARG ALA LEU ASP THR ASN TYR LYS PHE  
 1102 ATG ATT CCT CCA GAC CGG CTA GAC AAC CCA GGC CTG GGG GCT CAG AGG AAG AAG AAG CGG GCC CTG GAC ACC AAC TAC TGC TTC  
 280  
 ARG ASN LEU GLU GLU ASN LYS VAL ARG PRO LEU TYR ILE ASP PHE ARG GLN ASP LEU GLY TRP LYS TRP VAL HIS GLU  
 1183 CGC AAT TTG GAG GAG AAC TGC TGT GTG CGC CCT CTC TAC ATT GAC TTC CGA CAG GAT CTG GGC TGG AAG TGG GTC CAT GAA  
 310  
 PRO LYS GLY TYR TYR ALA ASN PHE LYS SER GLY PRO LYS PRO TYR LEU ARG SER ALA ASP THR THR HIS SER SER VAL LEU  
 1264 CCT AAG GGC TAC TAT GCC AAC TTC TGC TCA GGC CCT TGC CGG TAC CTC CGC AGT GCA GAC ACA ACC CAC AGC TCG GTG CTG  
 320  
 GLY LEU TYR ASN THR LEU ASN PRO GLU ALA SER ALA SER PRO LYS LYS VAL PRO GLN ASP LEU GLU PRO LEU THR ILE LEU  
 1345 GGG CTG TAC AAC ACC CTG AAC CCC GAA GCC TCG GGC TCT CCG TGC TGC GTC GTC CCC CAG GAC CTG GAG CCC CTG ACC ATC CTG  
 330  
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Fig. 1a(cont.) II

320  
 TYR TYR VAL GLY ARG THR ALA LYS VAL GLU GLN LEU SER ASN MET VAL VAL LYS SER LYS LYS SER  
 1426 TAC TAC TAC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC  
 1509 CA CCCCAGAGAG 6AGGAGAAAT 6CCACTGCTT 6CCTGCTGCTT 6CCTGCTGCTT 6CCTGCTGCTT 6CCTGCTGCTT 6CCTGCTGCTT  
 1608 CAG CTTCCACGCAA GTGGCCGAGAG CAGAGGTTCC CTTTGGGAAAC ATTTCCTTT CTTTCTGCTT CTTTCTGCTT CTTTCTGCTT  
 1707 TTA 6GSAAGGTTG AACTCTTTCAG AACACACGGA TTTTCTGCTT CTTTCTGCTT CTTTCTGCTT CTTTCTGCTT CTTTCTGCTT  
 1806 6GCCC ATGGGATTTG 6GATACCCAA 6GGAAGGAGGA AGGCGAGAGA ATGGCCAGGA CAGGCCCCAGA CTTGGAAGATG AGGTCAGATG GCTC  
 1905 ATGCCC 6CCCCAAGTC TCTCTAAGG 6ATCTGATTT ACGTCAATCA AGGCAAGTGT TTTTCTGCTT CTTTCTGCTT CTTTCTGCTT CTTTCTGCTT  
 2004 CTTATAC TTACCTGAGG TTAAGGACAA ATCTGTTAGT TTGCAATTT GTCCCATGGA CATCAGTTCAG CATCAGGCTT CACTACAGG AGAAATCCA 6G  
 2103 TAAGGTAG TTCCGCGGCC GTCCACTATA TTGCGCCCTA TGGATATGCT GAATCTAGGA GCAGAGGCTT GGAATTAAC CTTCTCTGTT CTTGCCCCCTG G  
 2202 GTTCTCTCT CTTGCTCTCT TCTTCTCTCT TATTTCTCTT TGGACATTTG GCTAGACACC TTCCAGGCTT CTTTCTGCTT CTTTCTGCTT CTTTCTGCTT  
 2302 AGCCCCGAG 6CATTAAGG TTTCTCTCTT TGAACCTCTT AAGACTTGT GCTCATCTG 6GTTCTCTGGA AGCAGGCTCT ACAACGCTG AGGCTGCTG G  
 2402 GAAATTCGA CAGGTGCTT ACAATGACTT GGGCCCCAGAT GCATAGACTT AGGTATAAG ACAATACAA ATATTACTCT CAAATATCTT TGTATAATA A  
 2502 ATATTTTTT 6GGAATCTT 6GATCATTTT ATCTCTGGA AGATTGTTT TAAACATAA AGGCTTATTT CTAAGGCTGTA AAAA

Fig. 1b.

1 CCTGTTTAA GACATGACAGG CCGTACAGG CACACAGTCC GCTTCTTGGT CCGACGGGT GCCAGCGCTT CCGGAGAGTC CTAAGGCTCT  
 201 CCGACTGCAG TGAATTCATG CACCTTCTTG CCAAGCCCTCA GTCTTGGGA TCTGGGAGAG CCGCTTGGTT TTTCTTCCCT CTTCTGACAG TCTCTGAGG  
 201 TCTCTTCCCT TCCAAGGCTT GCGGCTCCCT TGGCTCTCT TCCACCTCA CACAAGAG  
 30 MET HIS LEU GLN ARG ALA LEU VAL VAL LEU  
 ATG CAC TTG CAA AGG GCT CTG GTG GTC CTG  
 30  
 290 GGC CTG CTG AAC TTT GCC ACG GTC AGC CTC TCT CTG TCC ACT TGC ACC ACC TTG GAC TTC GGC CAC ATC AAG AAG AAG AGG  
 30 ALA LEU LEU ASN PHE ALA THR VAL SER LEU SER LEU SER THR ~~LYS~~ THR LEU LEU ASP PHE GLY HIS ILE LYS LYS ARG  
 371 GTC GAA GCC ATT AGG GGA CAG ATC TTG AGC AAG CTC AGG CTC ACC AGC CCC CCT GAG CCA ACG GTG ATG ACC CAC GTC CCC  
 50 VAL GLU ALA ILE ARG GLY GLN ILE LEU SER LYS LEU ARG LEU THR SER PRO PRO GLU PRO THR VAL MET THR HIS VAL PRO  
 70 TYR GLN VAL LEU ALA LEU TYR ASN SER THR ARG GLU LEU LEU GLU MET HIS GLY GLU ARG GLU GLU GLY ~~LYS~~ THR GLN  
 80 TAT CAG GTC CTG GCC CTG TAC AAC AGC ACC CCG GAG CTG CTG GAG GAG ATG CAT GGG GAG AGG GAG GAA GGC TGC ACC CAG  
 90  
 533 GAA AAC ACC GAG TCG GAA TAC TAT GCC AAA GAA ATC CAT AAA TTC GAC ATG ATC CAG GGG CTG CCG GAG CAC AAC GAA CTG  
 100 GLU ASN THR GLU SER GLU TYR TYR ALA LYS GLN ILE HIS LYS PHE ASP MET ILE GLN GLY LEU ALA GLN HIS ASN GLN LEU  
 110  
 614 GCT GTC TGC CCT AAA GGA ATT ACC TCC AAG GTT TTC CGC TTC AAT GTG TCC TCA GTG GAG AAA AAT AGA ACC AAC CTA TTC  
 120 ALA VAL ~~LYS~~ PRO LYS GLY ILE THR SER LYS VAL PHE ARG PHE ASN VAL SER SER VAL GLU LYS ASN ARG THR ASN LEU PHE  
 130  
 695 CGA GCA GAA TTC CGG GTC TTG CGG GTC CCC AAC CCC AGE TCT AAG CGG AAT GAG CAG AAG ATC GAG CTC TTC CAG ATC CTT  
 140  
 150 ARG ALA GLU PHE ARG VAL LEU ARG VAL PRO ASN PRO SER SER LYS ARG ASN GLU GLN ARG ILE GLU LEU PHE GLN ILE LEU  
 160  
 170



Fig. 1b(cont.) I

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 ARG PRO ASP GLU HIS ILE ALA LYS GLN ARG TYR ILE GLY GLY LYS ASP LEU PRO THR ARG GLY THR ALA GLU TRP LEU SER  
 776 CCG CCA GAT GAG CAC ATT GCC AAA CAG CCG TAT ATC GGT GGC AAG AAT CTG CCC ACA CGG GGC ACT GCC GAG TGG CTG TCC  
 190  
 220  
 200  
 PHE ASP VAL THR ASP THR VAL ARG GLU TRP LEU LEU ARG ARG GLU SER ASP LEU GLU ILE SER ILE HIS ARG PRO  
 857 TTT GAT GTC ACT GAC ACT CTG CCG GAG TGG CTG TTG AGA AGA GAG TCC AAC TTA GGT CTA GAA ATC AGC ATT CAC TGT CCA  
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*Fig. 1b(cont.)II*

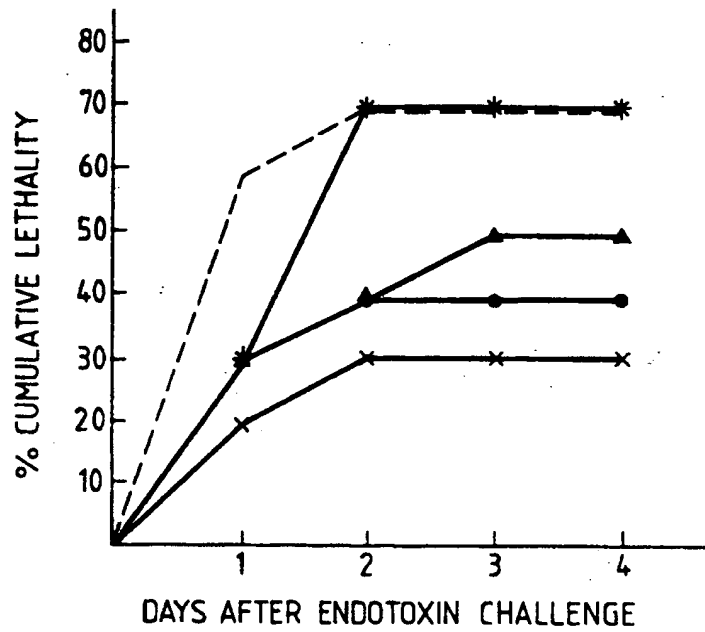
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 2202 CCTCTCTCT CACCTCTCCC TCGATCATAT TTCCCTTGG AACTTGGT AGACGCTTC CAGGTGAGGA TGCACATTTC TGGATGTGAG TTCCATGCGAG C  
 2302 CTGGGACA TTATGGGCT TCCCCACTT CCCCCCAAG ACCCTGTGTT CATTTGGTCT TCTTGGAGC AGGTGCTACA ACAGTGAGG CATTCGGGGA A  
 2402 GCTGACAT GTGCCACACA GTGACTTGG CCGAGAGGCA TAGACTGAGG TATAAGACA AGTATGAATA TTACTCTCAA ATCTTTGTA TAAATAAATA T  
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19

*Fig.2.*

$h.\beta\text{-TGF}_1$ :	ALDTNYCFSSTEKNCCVRQLYIDFRKDLGWK-WIHEPKGY
$h.\beta\text{-TGF}_3$ :	ALDTNYCFRNLEENCCVRPLYIDFRQDLGWK-WVHEPKGY
$p.\beta\text{-TGF}_3$ :	ALDTNYCFRNLEENCCVRPLYIDFRQDLGWK-WVHEPKGY
$b.\beta\text{-TGF}_2$ :	ALDAAAYCFRRVQDNCLRLPLYIDFKRDLGW-----
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$h.\beta\text{-TGF}_1$ :	HANFCLGPCPYIWSLDT----QYSKVLAL-YNQ--HNPGA
$h.\beta\text{-TGF}_3$ :	YANFCSGPCPYLRSADT----THSTVLGL-YNT--LNPEA
$p.\beta\text{-TGF}_3$ :	YANFCSGPCPYLRSADT----THSSVLGL-YNT--LNPEA
$b.\beta\text{-TGF}_2$ :	
	*                *
	40            50            60            70
$h.\beta\text{-TGF}_1$ :	SAAPCCVPQALEPLPIVYYV-GRKPKVEQLSNMIVRSCKCS
$h.\beta\text{-TGF}_3$ :	SASPCCMPQDLEPLTILYYV-GRTPKVEQLSNMVVKSCCKS
$p.\beta\text{-TGF}_3$ :	SASPCCVPDLEPLTILYYV-GRTAKVEQLSNMVVKSCCKS
$b.\beta\text{-TGF}_2$ :	
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	80            90            100            110

*Fig. 3.*



*Fig. 5.*

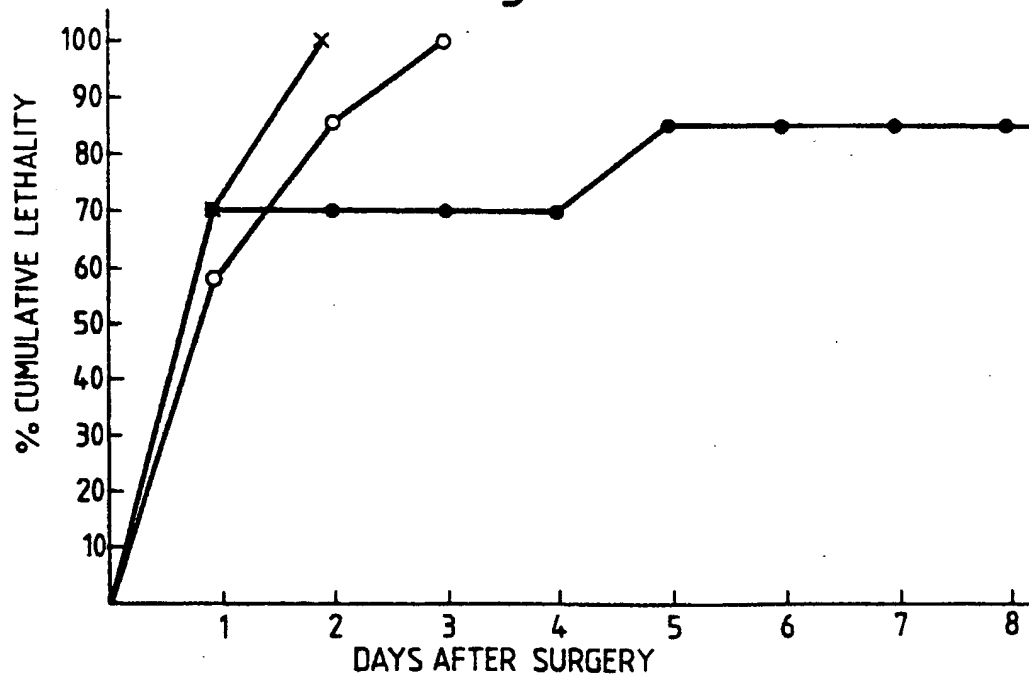


Fig. 4.

